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IRON OVERLOAD AND THE PREDISPOSITION OF CELLS TO ANTIOXIDANT CONSUMPTION AND PEROXIDATIVE DAMAGE

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We have investigated the effects of iron overload *in vivo* on the tocopherol levels and the extent of lipid peroxidation in rat liver microsomes and their response to subsequent oxidative stress *in vitro*. The results demonstrate a direct correlation between consumption of antioxidant defences and the induction and extent of malondialdehyde production in microsomes prepared from iron-loaded rats. The data are consistent with the requirement for iron (II)/iron (III) ratios in lipid peroxidation in control microsomes.

KEY WORDS: α-tocopherol, malondialdehyde, hplc, iron overload, oxidative stress.

INTRODUCTION

Clinical evidence for toxicity caused by iron excess has been provided by studies of patients with idiopathic haemochromatosis,[1] iron overload in Bantus[2] and secondary haemochromatosis due to repeated blood transfusion.[3] Several mechanisms whereby chronic iron overload induces cellular injury and functional abnormalities in hepatocytes have been postulated which are mutually inter-dependent: increased fragility of lysosomal membranes mediated by peroxidative damage,[4,5] iron- induced peroxidation of microsomal and mitochondrial membranes,[6] increased collagen biosynthesis.[7]

alpha-Tocopherol is the major lipid-soluble free radical scavenger in the rat liver.[8] Sies *et al.* have shown that in rat liver microsomal membranes stressed by exogenous NADPH/iron-ADP, the lag-phase preceding the onset of lipid peroxidation is substantially shortened in preparations from vitamin E-deficient rats.[9] When hepatic mitochondrial preparations from vitamin E-loaded rats were exposed to a transition metal-mediated radical flux, an increase in the lag-phase of lipid peroxidation was observed.[10]

In this study we have investigated the effects of iron overload *in vivo* on the antioxidant status in rat liver microsomes, the relationship to the extent of endogenous lipid peroxidation and the response of these parameters to subsequent oxidative stress *in vitro*. The results demonstrate a direct correlation between consumption of alpha-tocopherol and the formation of specific aldehydic secondary metabolites of lipid peroxidation in the microsomes prepared from rat livers that were iron-loaded *in vivo*. Furthermore, the data lend support to the proposal[11] for the

requirement of optimal iron II/iron III ratios in iron-induced lipid peroxidation in the control microsomes.

EXPERIMENTAL

All chemicals were purchased from either Sigma or BDH Chemicals except Sepharose 4B (Pharmacia), alpha-tocopherol (Roche Products) and desferrioxamine (CIBA-Geigy). All solvents were hplc grade and were obtained from Rathburns Chemicals (Peebleshire, Scotland). Iron contamination was removed from laboratory reagents using the high affinity iron-binding protein transferrin by a modification (McCarthy and Evans, unpublished work) of the method of Gutteridge.[12] Apo-transferrin was prepared from homogenised hen egg white[13] and coupled to agarose gel (Sepharose 4B) which had been activated by cyanogen bromide treatment.[14] All aqueous solutions were filtered through the gel prior to use.

Male rats (250 g) of Sprague-Dawley strain were iron-loaded by intramuscular injection with iron-dextran (Imferon, Fisons) at a dose of 100 mg/rat. Control rats were injected with 0.9% saline. Two weeks were allowed for the distribution of the iron in both the parenchymal and Kupffer cells. Urinary and faecal samples were then collected for assay of excreted iron over a period of 48 h, after which the rats were sacrificed.

Microsomes from livers of control and iron-loaded rats were prepared from 20% homogenates in cold 0.25 M sucrose which had been centrifuged at 10,000 × g_{av} for 10 min in the 50 mol rotor of a Sorvall centrifuge. They were aggregated with 5 vol of 6 mM calcium chloride/0.0125 M sucrose and sedimented in the cold at about 500 × g_{av} for 5 min, essentially according to Kamath and Rubin.[15] Pellets were stored at −70°C and were resuspended in 0.15 M KCl immediately before use. Protein concentration of microsomal membranes was assayed by the procedure of Lowry *et al.*[16] using bovine serum albumin (Sigma) as standard. Atomic absorption spectroscopy was applied to measure the iron levels of the excreted urine and faeces. Iron was detected at 248.3 nm using a Perkin-Elmer 3030 atomic absorption spectrometer. Samples were evaporated, resuspended in 2 ml of concentrated nitric acid and digested at 140°C for 20 min. Standards and blanks were incorporated.

Exogenous iron stress was initiated[17] by incubating microsomes (concentration 5 mg/ml) with 0.02 mM iron II/1.4 mM ADP/0.5 mM NADPH in buffer at pH 8.0 for up to 30 min. During the incubation, the suspension was stirred constantly, bubbled with 95% oxygen and kept at 25°C. At regular intervals, 1 ml aliquots of the suspension were withdrawn into tubes containing either an equal volume of acetonitrile or two volumes of ethanolic pyrogallol (2% w/v) before hplc assay of malondialdehyde and vitamin E respectively.

Malondialdehyde (MDA) was measured by a modification of the method of Esterbauer and Slater[18] and Esterbauer *et al.*[19] which was originally developed for studying MDA formation by rat liver microsomes. After brief centrifugation, 20 ul of the supernatant were injected onto a Spherisorb 5-aminocolumn (250 × 4.8 mm, Hichrom Ltd, UK) through a Rheodyne syringe loading injection valve fitted with a 20 ul loop (Alltech, UK). MDA was eluted using a mobile phase of 50% acetonitrile, 50% Tris buffer (0.03 M, pH 7.4) at ambient temperature and a flow rate of 1.0 ml/min. Detection was by UV absorbance at 270 nm using an SA 5000 variable wavelength detector (Severn Analytical). A malondialdehyde stock solution (10 mM)

(10 mM) was prepared by dilution of 1,1,3,3-tetraethoxy propane to a volume of 100 ml in sulphuric acid (1% v/v). After two hours, this solution was diluted one hundred fold with 1% acid and the concentration measured using the molar extinction coefficient at 245 nm of 13,700.

alpha-Tocopherol was measured by the method of Buttriss and Diplock *et al.*[20] after alkaline saponification, extraction in hexane and by adsorption hplc using a Lichrosorb Si-60 (150 × 4.8 mm) column and a mobile phase of methyl-t-butyl ether and hexane (8:92 v/v). A hexane solution of alpha-tocopherol of concentration 10 ug/ml was used for calibration of the assay.

RESULTS

A measure of the antioxidant status of the rat liver microsomes after iron overload *in vivo* was assessed by determining alpha-tocopherol levels. Levels in microsomes prepared from iron-loaded rats were only 63% of the normal control levels, at 0.137 ± 0.044 [n = 11] compared with 0.220 ± 0.034 ug/mg protein [n = 3]. Membranes were further stressed *in vitro* using iron II/ADP/NADPH and the subsequent alpha-tocopherol depletion on incubation at 37°C was measured as a function of time (Figure 1). The levels in the microsomes from iron-loaded rats decreased steadily as the time of incubation increased. This was in contrast with the control microsomes which displayed a delay in the onset of consumption of alpha-tocopherol and subsequently showed a slower rate of decline on prolonged incubation. After 5 min incubation with the exogenous iron stress the alpha-tocopherol levels had decreased to 64%

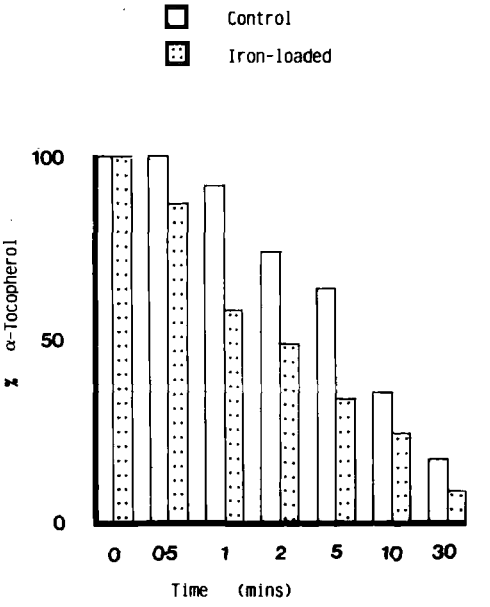

FIGURE 1 Alpha-tocopherol content of hepatic microsomal membranes from control and iron-loaded rats after subsequent exogenous stress with ADP/FE(II)/NADH. (The results are expressed as the percentage of the concentration measured before exposure to iron-stress *in vitro*).

and 34% of the original levels in the control and iron-loaded microsomes respectively.

As a marker for the susceptibility to lipid peroxidation, malondialdehyde levels were determined using high performance liquid chromatography of the microsomal membranes. The control microsomes showed a very low endogenous content of 0.83 ± 0.07 nmoles MDA/mg microsomal membranes protein [n = 3] which compare favourably with the reported data of Esterbauer.[21] Microsomes prepared from iron-loaded rats contained 2.96 ± 1.88 nmoles MDA/mg protein [n = 15]. On exogenous iron stress (Figure 2) the control microsomes slowly peroxidised whereas the iron-loaded samples responded immediately to the iron-stress peroxidising more readily, so that after 5 minutes incubation the levels were 11.4 ± 1.63 [n = 3] and 25.0 ± 8.3 [n = 15] nmoles MDA/mg protein respectively. No modifications in fatty acid composition were detected after *in vivo* iron-loading. This agrees with the observations of Buttriss and Diplock[22] who suggest a mechanism of replacement of oxidised fatty acids *in vivo* on oxidative stress.

For assessment of iron excretion after iron overload, urine and faeces were collected over a period of 48 hr. As expected, there were no significant differences in urinary or faecal iron excretion from the control and iron-loaded rats.

DISCUSSION

The occurrence of an induction phase preceding the onset of lipid peroxidation in control rat liver microsomal preparations stressed with NADPH/iron-ADP is well-documented.[23–25] This lag period has been attributed to the ability of the inherent antioxidant defence mechanisms in the membrane to break the propagation of the chain of lipid peroxidation, which does not proceed until the antioxidant defences are substantially depleted.[26] It has been proposed that the duration of the lag period is related to the nature and effectiveness of endogenous and exogenous antioxidants[9]

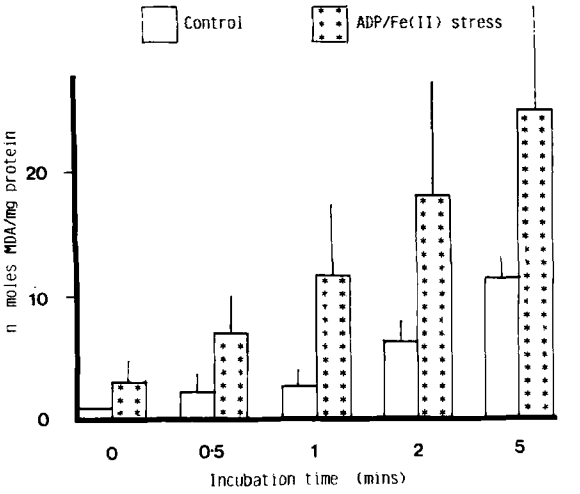

FIGURE 2 MDA levels in hepatic microsomal membranes from control and iron-loaded rats after subsequent exogenous stress with ADP/FE(II)/NADH.

and, as the most significant membrane antioxidant, alpha-tocopherol has a central role.

The sequence of equations below shows the fate of a propagating lipid peroxy radical in the membrane in the vicinity of an iron complex:[27]

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

$$LOO^{\cdot} + \text{alpha-TH} \rightarrow LOOH + \text{alpha-T}^{\cdot}$$

$$LOOH + \text{Fe(II)-complex} \rightarrow LO^{\cdot} + \text{Fe(III)-complex} + OH^{-}$$

$$LO^{\cdot} + \text{alpha-TH} \rightarrow LOH + \text{alpha-T}^{\cdot}$$

During this process, lipid peroxy radicals are converted, mediated by the catalytic iron (II)-complex, to the end product LOH and to iron (III). Hence, although lipid hydroperoxides are converted, during this sequence of reactions, to end product secondary alcohols, the formation of an iron (III)-complex can lead to the continual regeneration of lipid hydroperoxide, LOOH.

$$LOOH + \text{Fe(III)-complex} \rightarrow LOO^{\cdot} + \text{Fe(II)-complex} + H^{+}$$

$$LOO^{\cdot} + \text{alpha-TH} \rightarrow LOOH + \text{alpha-T}^{\cdot}$$

For each complete redox cycle of one mole of iron (II)-complex via this sequence, one mole of peroxy radicals is terminated, one mole of hydroperoxide is formed and two moles of alpha-tocopherol are consumed.

This general mechanism can only operate where the antioxidant is localised in close proximity to the polyunsaturated fatty acyl chains. Not all unsaturated fatty acids in the membrane may have access to alpha-tocopherol.[28] In rat liver microsomal membranes the molar ratio of polyunsaturated fatty acids to tocopherol is several orders of magnitude (from our studies)[22] which is in close agreement with the ratios reported by Fukuzawa *et al.*[29] However, there is now evidence that the diffusion coefficient of alpha-tocopherol in membranes may be quite high which could account for the apparent ability of one mole of tocopherol to quench radicals formed in several moles of polyunsaturated fatty acids.[30] The slow decline in the tocopherol levels of the control microsomes on exogenous iron-stress presented here may reflect its initial inaccessibility.

Aust *et al.*[11] have implicated the importance of ferrous:ferric ratios in the initiation of iron-mediated lipid peroxidation and their significance in the time-dependency of the onset of such peroxidative events in systems containing no pre-formed lipid hydroperoxides. His studies attributed the lag period to the time required to achieve a catalytic ratio of oxidised and reduced species. The initial slow decline in alpha-tocopherol in the control microsomes observed in our studies on exogenous iron stress can be interpreted as being consistent with the time required for the generation of suitable initiating species before the antioxidant role of tocopherol is required.

In the microsomes from the rats in which iron overload was induced *in vivo*, the depletion of 40% of the total alpha-tocopherol was enough to promote the complete abolition of the lag phase in the lipid peroxidation on subsequent exogenous iron stress and suggests the formation of lipid hydroperoxides *in vivo*. This can be compared to a fall in the rat liver microsomal level of alpha-tocopherol to 20% of the control value following the feeding of a vitamin E-deficient diet for 16 weeks.[25] The

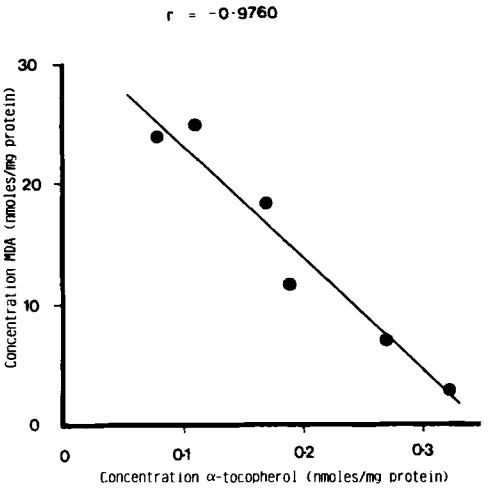

FIGURE 3 The relationship between alpha-tocopherol and MDA levels in hepatic microsomal membranes from iron-loaded rats when the fractions were subsequently exposed to exogenous iron-stress *in vitro*.

major difference between iron overload *in vivo* and tocopherol deficiency *in vivo* may be the timescale of formation of lipid hydroperoxides.

The further rapid tocopherol depletion following exogenous iron stress parallelled the generation of the specific aldehydic metabolite of lipid hydroperoxides, malondialdehyde, as indicated in Figure 3, with a correlation coefficient of -0.976. This suggests that iron overload *in vivo* predisposes liver microsomal preparations to antioxidant consumption and lipid peroxidation results.

Studies by Bacon *et al.*[6] have examined hepatotoxicity induced by feeding rats diets supplemented with iron-carbonyl, which preferentially loads hepatocytes rather than the reticulo-endothelial system. Their results have shown that this causes the appearance of conjugated dienes in lipids extracted from both hepatic microsomes and mitochondria as well as a 65–75% decrease in hepatic alpha-tocopherol concentrations. This decrease is tentatively ascribed to the destruction of the vitamin in the GI tract lumen by dietary iron, which might in part account for the lesser (37%) decrease seen in our studies when rats were loaded parenterally.

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